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Comparison of Animal Infectivity and Excystation as Measures of *Giardia muris* Cyst Inactivation by Chlorine

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In this study, in vitro excystation and mouse infectivity were compared as methods for quantitatively determining the viability of *Giardia muris* cysts before and after exposure to free residual chlorine. The mouse infectivity results show that very few cysts (1 to 15) constitute an infectious dose. The results of the inactivation studies indicate that in vitro excystation is an adequate indication of *G. muris* cyst infectivity for the host and can be used to determine the effects of disinfectants on cyst viability.

Over the past several years, giardiasis has consistently been one of the most frequently occurring waterborne diseases transmitted by drinking water in the United States (3). Waterborne transmission occurs via the cyst stage of the etiologic agent *Giardia lamblia*. Most outbreaks occur in drinking water systems that rely only on disinfection for treatment. To assess the adequacy of this treatment, information on the effects of disinfectants on the viability of cysts is needed.

The development of in vitro methods for measuring cyst viability (2, 12) has made it possible to determine the effects of chlorine (5, 11) and other disinfecting agents (6, 10) on cyst viability. Difficulties encountered in obtaining adequate supplies of *G. lamblia* cysts for such studies led to the use of a model cyst system, employing the cysts of *Giardia muris*, which are infective for mice (13). Modified excystation procedures for *G. muris* cysts have been developed (14) and comparative disinfection data on *G. lamblia* and *G. muris* have been reported for chlorine (11) and other disinfectants (8, 18). The adequacy of in vitro excystation for measuring loss of animal infectivity of cysts after exposure to disinfectants has not been established. This study was undertaken to compare the two methods and, thus, to assess the validity of in vitro excystation as an indicator of cyst inactivation by disinfectants.

G. muris cysts (initially isolated from infected mice supplied by David Stevens, Department of Medicine, University Hospital, Cleveland, Ohio) were produced in outbred female Swiss albino mice (CF-1). These cysts were used as the initial inoculum and were subsequently passaged at weekly intervals. Cysts present in fresh stools obtained from infected mice were separated by flotation on 1 M sucrose, washed twice by centrifugation, and stored in distilled water at 5°C. Cyst densities were determined by hemacytometer count and adjusted to appropriate concentrations for use in the disinfection experiments.

All disinfection experiments were conducted at 5°C at pH 7.0 and a free chlorine residual of 1 mg/liter. Stock chlorine solutions were prepared from sodium hypochlorite as described previously (11). Chlorine determinations were done at the beginning of each experiment and at the end of each exposure time using the *N,N*-diethyl-*p*-phenylenediamine colorimetric method (1). Chlorine levels remained constant throughout the course of the experiments. The disinfection experiments were conducted in mechanically stirred beakers immersed in a water bath at 5°C. To 500-ml quantities of chlorine-demand-free buffer containing 1 mg of free residual chlorine per liter, 5×10^6 washed cysts contained in 1 ml of distilled water were added, to provide a final cyst concentration of 1×10^3 cysts per ml. Control beakers containing 500 ml of unchlorinated buffer were dosed with cysts at the same level. At appropriate time intervals, chlorine action in individual beakers was stopped by the addition of 1 ml of 10% sodium thiosulfate. The sodium thiosulfate solution was also added to the control beakers. The cysts contained in each 500-ml volume were then reconcentrated by filtration through a 5.0-μm-pore-size, 47-mm polycarbonate filter under light vacuum. The cysts were washed from the filters with 2.0 ml of a 0.01% aqueous solution of polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co., St. Louis, Mo.) using a Pasteur pipette. The cyst densities in the reconcentrated suspensions were determined by hemacytometer count and divided into 0.5- and 1.5-ml portions for in vitro excystation and mouse infection, respectively. The in vitro excystations were accomplished in the initial experiments by the method of Bingham and Meyer (2) and in the later experiments by a modified excystation procedure (14).

In the modified procedure, excystation was induced by exposure to 1× Hanks solution supplemented with 17 mM glutathione–29 mM L-cysteine hydrochloride–50 mM sodium bicarbonate at pH 2.0 for 30 min at 35°C. This was followed by washing and suspension in trypsin-Tyrodes solution at pH 8.0. After processing through the excystation procedure, depression slides filled with cyst suspensions and sealed by vaspar (a mixture of equal parts of vaseline and paraffin)-lined cover glasses were inverted and incubated at 37°C for 30 min. After incubation, slides were examined microscopically with a Zeiss inverted phase-contrast microscope. Full (unexcysted) and empty (excysted) cysts were counted, and percent excystation of chlorine-exposed and control cysts was determined. The total number of cysts observed and scored as full or empty ranged from 200 to 1,000 in individual experiments. Percent excystation of control cysts was corrected to 100% and percent excystation of chlorine-exposed cysts was then corrected by the same factor as follows: [100/percent observed excystation (control cysts)] × percent observed excystation (exposed cysts) = corrected percent excystation (exposed cysts). Percent inactivation of the exposed cysts was then calculated as follows: percent inactivation = 100 − corrected percent excystation (exposed

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TABLE 1. Comparison of viability of *G. muris* cysts as determined by excystation and mouse infectivity

Expt no.	No. of cysts/ID_{50}	% Excystation
1	7.94	72[a]
2	10.0	77[a]
3	2.51	79[a]
4	15.85	95[b]
5	3.98	95[b]
6	1.26	95[b]
7	3.98	91[b]
8	0.13	99[b]
9	2.0	99[b]
10	2.51	97[b]
Mean	5.02	

[a] Bingham and Meyer excystation method (2).
[b] Modified excystation method (14).

cysts). For each experiment, the chlorinated sample showing ~90% or more cyst inactivation by excystation was selected for determination of mouse infectivity by determination of the 50% infective dose (ID_{50}).

For the animal infectivity studies, the cyst concentration of the selected reconcentrated chlorine-exposed cyst suspension was adjusted to contain 5×10^5 cysts per ml. Serial decimal dilutions were prepared to give inocula of 0.2 ml containing 10^5 (undiluted), 10^4, 10^3, 10^2, 10^1, and 10^0 cysts per mouse. Ten mice were each inoculated per os (by intubation with a 22-gauge feeding cannula) with each dilution. Each mouse was caged individually. Individually caged negative control mice were evenly dispersed among the other cages to check for intercage contamination. Control cyst suspensions, not exposed to chlorine were prepared in a similar manner and inoculated into mice at doses of 10^2, 10^1, 10^0, 10^{-1}, and 10^{-2} cysts per mouse. ID_{50} titers of control and chlorine-exposed cyst suspensions were determined by using the Spearman-Karber (4) method. Since the number of cysts in each dose was known through microscopic count, the actual number of cysts constituting one ID_{50} could be calculated. The percentage of cysts inactivated by chlorine was determined by dividing the number of control cysts needed for one ID_{50} by the number of exposed cysts required for one ID_{50}.

Unexposed cyst viability (as determined by percent excystation) is compared with mouse infectivity (as determined by number of cysts per ID_{50}) in Table 1. It is evident that excystation percentages were higher using the modified procedure (14) than when using the method of Bingham and Meyer (2), which was originally developed for *G. lamblia* excystation. The percent excystation attained with the modified method ranged from 91 to 99%. In this same set of samples, the number of cysts required for one mouse ID_{50} ranged from 0.13 to 15.85 cysts, with a mean of 5.02 cysts. No relationship between percent excystation and number of cysts per ID_{50} is evident. The ID_{50} results show greater variability than the excystation results because of larger error terms inherent in the ID_{50} method.

The *G. muris* cyst infectivity data are in good agreement with the results of earlier much more limited studies involving feeding of *G. lamblia* cysts to humans (9). These data indicated that the *G. lamblia* ID_{50} for humans was ≤10 cysts. More recent studies with *G. muris* cysts (15), also based on very limited data, also indicated an infectious dose of ~10 cysts. Thus, our results substantiate the concept that the minimal infectious dose for these pathogens is very low.

The results of a series of seven experiments comparing cyst inactivation by chlorine as determined by excystation and by mouse infectivity are shown in Table 2. The results show that the exposure to chlorine under the experimental conditions used resulted in similarly large reductions in both mouse infectivity and percent excystation. The percent inactivation ratios (excystation/infectivity) of the two methods are near unity, and neither method consistently showed a higher degree of inactivation than the other method. Kasprzak and Majewska (7), in studies of *G. muris* survival in stored water, pointed out that the *G. muris* system, because of the higher in vitro excystation percentages attainable, offered a good approach to the in vitro determination of *Giardia* cyst viability. Some of their data, however, based on cyst infectivity for rats, indicated that even when in vitro excystation levels were zero, infection of some rats occurred. Their studies did not involve the use of disinfectants.

Stringer (16) developed a viability assay based on in vitro excystation for *Entamoeba histolytica*. He noted the advantages of this assay system over others used earlier, including staining methods that lacked specificity and quantal assay culture techniques that were tedious and time consuming. In later, more extensive studies, Stringer et al. (17) used the excystation method to determine the effectiveness of halogen disinfectants for inactivation of *E. histolytica* cysts. To our knowledge, excystation and animal infectivity for determining viability of *E. histolytica* cysts exposed to disinfectants have not been compared. The *E. histolytica* data apparently were implicitly accepted and their validity borne out by field experience.

The excystation bioassay system offers a simple, reliable method for determining percent viability in a population of

TABLE 2. Comparison of excystation and mouse infectivity for determining *G. muris* cyst inactivation by chlorine

Expt no.	Exposure time (min)	No. of cysts/ID_{50}		% Excystation		% Inactivation determined by:		Ratio of excystation/ infectivity
		Control	Chlorine exposed	Control	Chlorine exposed	Excystation	Increase in no. of cysts/ID_{50}	
3	20	2.51	12.59	79 (100)[a]	7 (8.9)[a]	91.1	80.1	1.14
4	30	15.85	79.43	95 (100)	11 (11.6)	88.4	80.0	1.24
5	60	3.98	316.23	97 (100)	6 (6.2)	93.8	98.7	0.95
6	60	1.26	31.62	95 (100)	11 (11.6)	88.4	96.0	0.92
8	60	0.13	50.12	99 (100)	3.6 (3.6)	96.4	99.7	0.97
9	60	2.00	25.12	99 (100)	4.1 (4.1)	95.9	92.0	1.04
10	60	2.51	12.59	97 (100)	4.1 (4.2)	95.8	80.1	1.20

[a] Corrected (see text).

cysts in contrast to animal infectivity bioassays, which are much more complex, expensive, and time consuming. The results of our study provide substantial support for the validity of using excystation data to indicate the inactivation of *Giardia* cysts by drinking-water disinfectants such as chlorine.

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